ANTIMICROBIAL ACTIVITY OF IRON OXIDE NANOPARTICLES

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF

Master of Science

In

Life Science

By

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Under The Supervision of

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2014

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CERTIFICATE

*This is to certify that the thesis entitled "**Antimicrobial activity of Iron Oxide nanoparticles**" submitted by **Ms. Sweta Pal** (Roll No: 412LS2048) in partial fulfilment of the requirements for the award of **Master of Science in Life Science** to the National Institute of Technology, Rourkela, is an authentic and original record of research work carried out by her under my supervision and guidance.*

To the best of my knowledge, the work incorporated in this thesis has not been submitted elsewhere for the award of any degree.

Place: Rourkela

Date: 11th/May/2014

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DECLARATION

I hereby declare that the thesis entitled **"Antimicrobial activity of Iron oxide nanoparticles"** submitted to Department of Life Science, National Institute of Technology, Rourkela for the partial fulfilment of the requirements for the degree of master of science in life science is an original piece of research work done by me under the guidance of Dr. Suman Jha, Assistant Professor, Department of Life Science, National Institute of Technology, Rourkela. No part of this work has been done by any other research person and has not been submitted for any other purpose.

Ms. Sweta Pal

412LS2048

ACKNOWLEDGEMENT

I take the privilege to express my utmost gratitude to my guide Dr. Suman Jha, Assistant Professor, Department of Life Science, National Institute of Technology, Rourkela for his excellent guidance, care, patience and for providing me with every facility to complete my dissertation. I am also grateful to my faculty members Dr. Bibekanand Mallick, Dr. Sujit Kumar Bhutia, Dr. Rasu Jayabalan and Dr. Surajit Das for their constant encouragement throughout my dissertation. I am grateful to Dr Samir Kumar Patra, HOD, Department of Life Science, National Institute of Technology, Rourkela for his moral support and valuable advice that kept me motivated throughout my work.

My heartiest thanks to Mr. Manoranjan Arakha, PhD Scholar, Department of Life Science, National Institute of Technology, Rourkela, for his care, co-operation, patience and timely advice in each and every step of my work. My special thanks to Mr. Parth Sarthi Nayak and Miss Shreyasi Asthana (PhD Scholars of Protein Biology Lab) Department of Life Science, National Institute of Technology, Rourkela for their timely help, good wishes and moral support during my dissertation.

I would also like to thank all my classmates and my lab mates for their help and support during my work. Last but not the least, I express my heartiest devotion to my beloved parents for their ethical and moral support, love and blessings which helped in the successful completion of my dissertation.

I bow my head before the Almighty for his blessings on me.

CONTENTS

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ABBREVIATION

NPs: Nanoparticles

IO: Iron oxide

DLS: Dynamic light scattering

XRD: X-ray diffraction

SEM: Scanning electron microscope

ATR-FTIR: Attenuated Total Reflection Fourier transform infrared spectroscope

Hrs: Hours

mins: Minutes

mV: Millivolt

nm: Nanometer

rpm: Rotation per minute

Abstract

The production and use of iron oxide nanoparticles are growing exponentially due to its wide applications in different fields of science and technology. The objective of this study is to synthesize iron oxide nanoparticles, and modification of its surface to make it less toxic. The iron oxide nanoparticles were synthesized by chemical co-precipitation method, and surface modification was done using chitosan. The synthesized nanoparticles were characterized using different techniques like FE-SEM, UV-Vis, DLS, Zeta analyzer, XRD and ATR-FTIR spectroscopes. The toxicity of iron oxide nanoparticles and modified iron oxide nanoparticles was evaluated against *Bacillus subtilis* and *Escherichia coli*, and as a conclusion it was found that iron oxide nanoparticles have toxicity towards these test organisms at higher concentration, where as modified iron oxide nanoparticles doesn't possess this property. The work confirms that toxicity of nanoparticle can be modulated by surface modification with biocompatible compounds, like chitosan.

INTRODUCTION

Nanoparticles are the simplest form of structures with sizes in the range of 1-100 nm. In principle any collection of atoms bonded together with a structural radius of less than 100 nm can be considered as a nanoparticle. They are a link between bulk materials and atomic or molecular structures. Nanoparticles have different chemical and physical properties than bulk materials such as lower melting points, higher surface area, mechanical strength, specific optical properties and specific magnetizations [1]. In the presence of chemical agents the interfacial and surface properties can be modified. Indirectly, such agents can stabilise against coagulation and aggregation by maintaining particle charge and by modifying the outermost layer of the particle. In the past decade, the synthesis of iron oxide nanoparticles has been intensively developed not only for its fundamental scientific interest but also for many technological and biomedical applications [2].

Iron oxide nanoparticles have been of great interest, not only for fundamental properties caused by their multivalent oxidation states but also for their super paramagnetic, high force, low Curie temperature, high magnetic susceptibility, etc. [3, 4]. Release of iron oxide nanoparticle into the environment interact with air, water and soil often causes change in the surface properties of the particles which can result in particle aggregation or changes in particle charge and other surface properties [5] . Various surface modifications are being done for making these non biodegradable nanoparticles more biocompatible. The iron oxide nanoparticles must be pre-coated with chitosan which is a biopolymer that increases their stability, biodegradability, and non-toxicity in the physiological medium and also to achieve combined properties of high magnetic saturation, biocompatibility and interactive functions on the surface [6].

Interaction mechanisms between nanoparticles and living systems are not yet fully understood. To understand the interaction mechanism we have to test for the antimicrobial activity of both iron oxide nanoparticle and modified iron oxide nanoparticle.

1. LITERATURE REVIEW

Nanoscience and technology is an interdisciplinary and broad area of research and development activity that has been growing dynamically worldwide in the past few years. Nanomaterials have wide-range applications and indications in a variety of areas, including chemistry, physics, electronics, materials science, optics and biomedical sciences. The nanomaterials exhibit solitary and considerably changed physical, chemical and biological properties, when compared to their macro-scaled, i.e. bulk compliments. The nanoparticle interaction with biological materials lead to the formation of new nanomaterial with control size, shape, surface chemistry, roughness and surface coatings [7]. Antimicrobial agents are of great importance in several industries such as water disinfection, packaging, textiles, construction, medicine and food [8].

The organic compounds traditionally used for disinfection produce several disadvantages, including toxicity to the human body, and sensitivity to high temperatures and pressures that are present in many industrial processes. For these reasons, the interest in inorganic disinfectants such as metal oxides is increasing [9]. These inorganic compounds present strong antimicrobial activity at low concentrations. They are also much more stable in extreme conditions considered as non-toxic, and some of them even contain mineral elements essential to the human body.

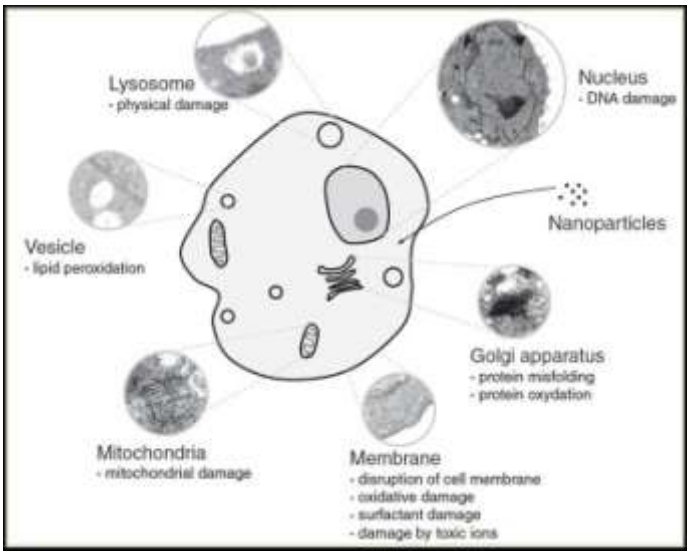

Figure 1: Nanoparticle interaction with Eukaryotic cell [5].

1.1. Iron oxide nanoparticle:

Iron oxide nanoparticles were synthesized by cost effective co-precipitation method. It possesses strong ferromagnetic behaviour and less sensitivity to oxidation. Iron oxide nanoparticles have attracted much interest because they belong to the class of materials having non-toxicity and biological compatibility due to the presence of Fe (II/III) ions [10].

Due to its 4 unpaired electrons in 3d shell, an iron atom has a strong magnetic moment. Ions Fe^{2+} has also 4 unpaired electrons in 3d shell and Fe^{3+} has 5 unpaired electrons in 3d shell. Therefore, when crystals are formed from iron atoms or ions Fe^{2+} and Fe^{3+} they can be in ferromagnetic, antiferromagnetic or ferrimagnetic states [11].

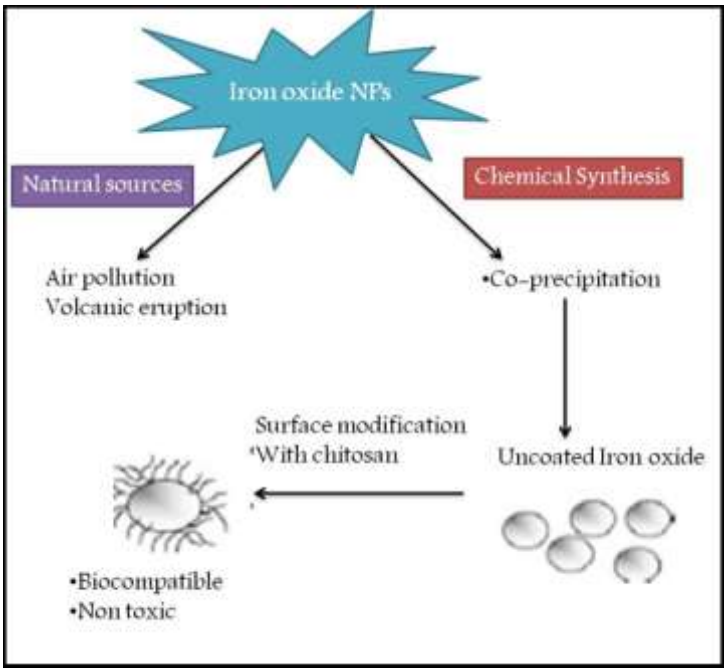

Figure 2: Iron oxide synthesis by co-precipitation method.

1.2. Iron oxide nanoparticles and the prokaryotic cell:

Unicellular organisms, which ruled the Earth for approximately the first 3 billion years of life by engulfing matter, usually particulate, from their immediate environment. There are the two basic mechanisms endocytosis and phagocytosis [5]. The phagocytosis is generally for large particles and requires a "recognition" step while the endocytosis is for the trans-membrane transport of liquids and molecules [12].

Mechanisms on the nano-bio interface can be either physical or chemical. Chemical mechanisms include the production of reactive oxygen species (ROS), termination and release of toxic ions, oxidative damage through catalysis, disturbance of the ion cell membrane transport activity and lipid peroxidation or surfactant properties. ROS is considered as being the main rudimentary chemical process in nanotoxicology which can lead to secondary processes that can ultimately cause cell damage and even cell death. Moreover, ROS is one of the main factors involved in inflammatory processes. This is assumed to happen via up-regulation of genes involved in the pro-inflammatory response stimulated by

the activation of certain transcription factors (NF-κB, AP-1). However, free radical formation can also have direct impacts on cell stability.[13, 14]

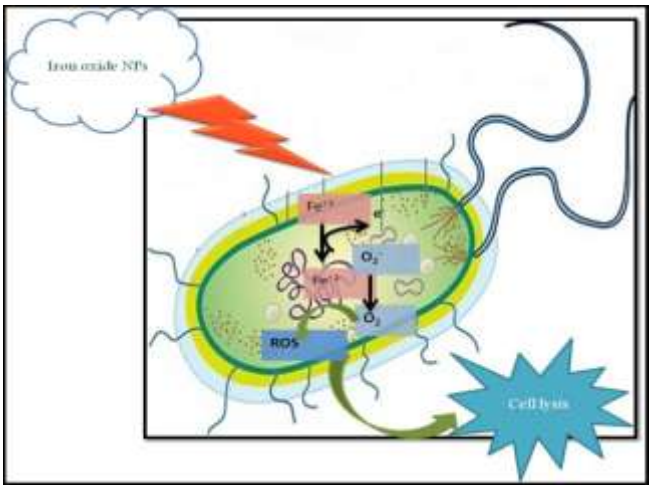

Figure 3: Nanoparticle interaction with Prokaryotic cell

Physical mechanisms at the nano-biointerface are mainly a result of particle size and surface properties. This includes membrane activity, disruption of membranes transport processes, protein conformation or folding and protein aggregation [15].

1.3. Surface modification of iron oxide nanoparticle:

The recent developments of nanotechnology in synthesizing biocompatible and functionalized magnetic nanoparticle have numerous applications in various fields. Chitosan is a partially acetylated glucosamine, poly (1 → 4)-2-amino-2-deoxy-d-glucan biopolymer. It is made by treating shrimp and other crustacean shells with the alkali sodium hydroxide [16]. It is a polyaminosaccharide with biodegradable, biocompatible and bioactive properties. With chitosan coating the surface of iron oxide nanoparticle become positively charged.

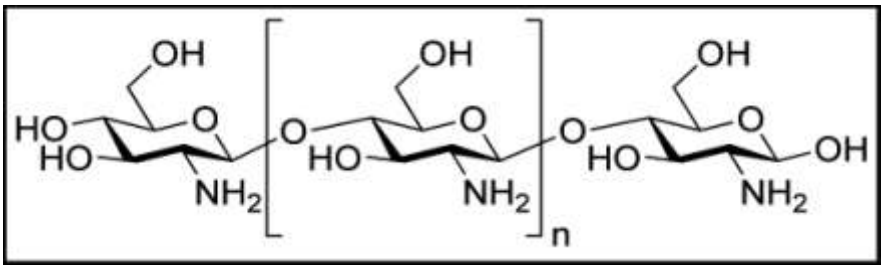

Figure 4: Structure of Chitosan

3. OBJECTIVES

Based upon the literature survey the current work has been proposed with the following objectives:

1. Synthesis of iron oxide nanoparticle by chemical co-precipitation method and its detail characterization

2. Surface modification of iron oxide nanoparticles

3. Evaluation of toxicity of iron oxide and modified iron oxide nanoparticles against *Bacillus subtilis* and *Escherichia coli*

4. MATERIALS AND METHODS

4.1. Chemicals:

For the synthesis of iron oxide nanoparticles, iron chloride tetra hydrate ($FeCl_2.4H_2O$) and iron chloride hexahydrate ($FeCl_3.6H_2O$) were purchased from sigma Aldrich Pvt Ltd. NaOH, nutrient broth and chitosan were purchased from HIMEDIA, India.

4.2. Glassware and Apparatus:

All glass wares such as measuring cylinders, test tubes, conical flasks, and beakers etc. were purchased from Borosil, India.

4.3. Bacterial Strains:

The test organisms *Bacillus subtilis* (MTCC.736) and *Escherichia coli* (MTCC 443) were purchased from Institute of Microbial Technology (IMTECH), Chandigarh, India, and maintained constantly on nutrient agar slant for further use.

4.4. Synthesis of Iron oxide nanoparticles:

The Iron oxide nanoparticles were synthesized by the co-precipitation of Iron chloride tetra hydrate ($FeCl_2.4H_2O$) and Iron chloride hexahydrate ($FeCl_3.6H_2O$). Iron chloride tetra hydrate ($FeCl_2.4H_2O$) and Iron chloride hexahydrate ($FeCl_3.6H_2O$) solutions were prepared in 100 ml of deionised water maintaining the molar concentration 0.1M and 0.2M respectively. The solution was mixed in a beaker and then stirred in a magnetic stirrer to get homogeneous solution. The solution was sealed and heated at 60 ^oC for 15-20 min in a water bath. Then 14 ml of Sodium hydroxide (NaOH) was added to the previous solution. A black precipitate was formed after the completion of the reaction. The precipitate was centrifuged at 7000 rpm for 15 min and pellet was collected. Then the pellet was dried at 50 ^oC to get the powder form of Iron oxide nanoparticles.

4.5. Surface modification of iron oxide nanoparticles:

Surface modification is a method to improve the surface characteristics of iron oxide nanoparticles. Surface modification is used to prevent aggregation, enhances the compatibility of iron oxide nanoparticles with biological environment and also improves the stability in suspensions. For surface modification chitosan was dissolved in 1 M of acetic acid with the final volume of 100 ml. Then it was mixed properly by magnetic stirrer for 15 mins followed by addition of 20 grams of chitosan and stirred for 30 mins. Then 70 mg of iron oxide nanoparticle was added and mixed for 18 hrs in the magnetic stirrer. A homogenous dark brown suspension was obtained. After 18 hrs, the sample was centrifuged at 7000 rpm for 15 mins and the pellet was collected and dried in a hot air oven.

4.6. Characterization of iron oxide nanoparticles:

4.6.1. UV-Vis spectroscopic analysis:

The synthesized iron oxide nanoparticles were characterized by UV-Vis spectrophotometer (Carry 100, Agilent, USA) to know the surface Plasmon resonance property of the nanoparticles. First the powdered form of the iron oxide nanoparticles were mixed in deionized water and then the sample was vortexed and analysed for the optical properties of iron oxide nanoparticles.

4.6.2. DLS particle size analysis:

Dynamic light scattering is a technique to determine the size distribution of particles in solution. It was employed to study the particle size of iron oxide nanoparticles. The prepared sample was dispersed in deionized water followed by sonication and then the particle distribution in liquid was studied in DLS (Malvern, Zeta Analyser)

4.6.3. Zeta potential analysis:

Zeta potential analyzer (Malvern Zeta Analyzer) was used to measure the potential difference between the dispersed medium and the stationary layer of fluid attached to the dispersed

particle. For zeta potential analysis the sample was mixed in deionized water and filtered by using 220 nm filter paper.

4.6.4. FTIR analysis:

Fourier transform infrared spectroscopy (FTIR) gives information about the vibrational and rotational modes of motion of a molecule and hence an important technique for identification and characterisation of a substance. For FTIR analysis of iron oxide nanoparticles, the dried powder was dispersed in deionized water and characterized by ATR-FTIR (Bruker, Germany) in the range 4000-500 cm^{-1}.

4.6.5. Scanning electron microscopy:

The morphological features of chemically synthesized iron oxide nanoparticles were studied by using FE-SEM (FEI NOVA Nano SEM).

4.6.6. XRD analysis:

X-ray diffraction (Rigaku, ultima iv) was used to study the phase variety and grain size of the iron oxide nanoparticles.

4.7. Growth kinetic study by iron oxide and modified iron oxide nanoparticles:

The growth kinetic study of *Bacillus subtilis* and *Escherichia coli* was done using different concentrations of Iron oxide and modified Iron oxide nanoparticles. Different concentrations of nanoparticles were added at mid log phase of bacteria and kinetic study was performed at regular time interval of 1 hour by using plate reader (Carry 100, Agilent, USA).

4.8. Sample preparation for Phase contrast microscopy:

Cultures of *B. subtilis* and *E. coli* were started and at the mid log phase nanoparticles were added and kept for overnight growth. One drop of culture was taken and put on the glass slide for phase contrast microscopic study.

5. RESULTS AND DISCUSSION

5.1. Synthesis of Iron oxide nanoparticles:

Figure 5 shows the iron oxide nanoparticles synthesized by chemical precipitation method. The powder form of synthesized nanoparticles is black in colour.

Figure 5: Chemically synthesized Iron oxide Nanoparticle.

5.2. Surface modification of Iron oxide nanoparticles:

Figure 6 shows the modified Iron oxide nanoparticles which are achieved using a biocompatible polymer called chitosan. When Iron oxide nanoparticle is added to chitosan solution it becomes brown in colour.

Figure: 6. Chitosan modified Iron oxide nanoparticle

5.3. Characterization of Iron oxide nanoparticles:

5.3.1. UV-Visible spectroscopy:

Figure: 7. shows the UV-Visible spectra of iron oxide nanoparticles and chitosan modified iron oxide nanoparticles. Iron oxide nanoparticle shows the peak at 222 nm where as chitosan modified iron oxide nanoparticle shows peak at 268 nm. The shifting of peaks confirms the coating of chitosan on the surface of Iron oxide nanoparticles.

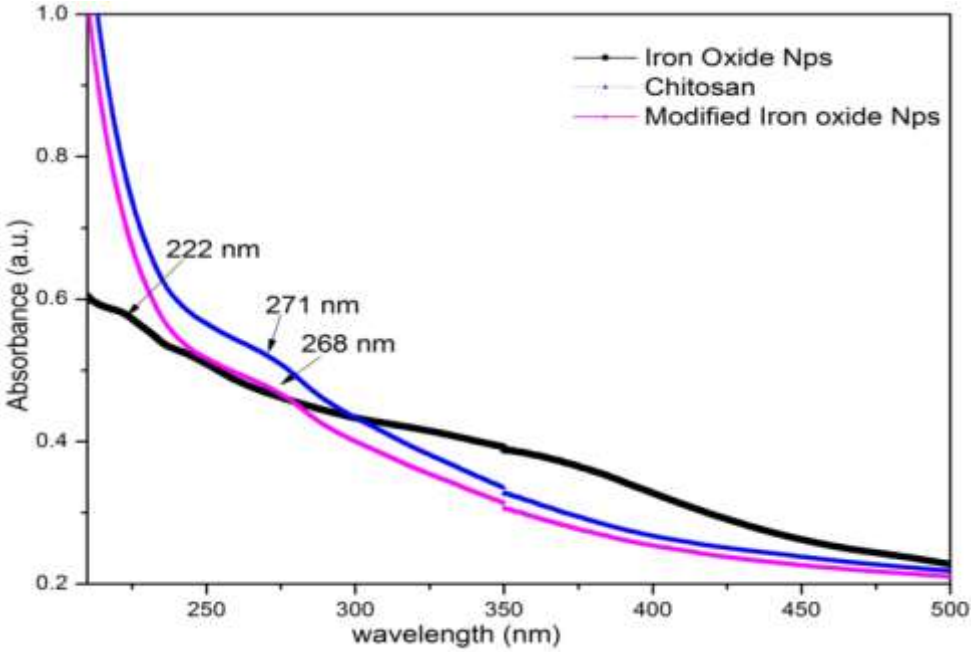

Figure 7: UV image of iron oxide nanoparticle and chitosan modified iron oxide nanoparticle.

5.3.2. DLS Analysis:

The particle size distribution of chemically synthesized iron oxide nanoparticles are shown in Figure: 8. and surface modified iron oxide nanoparticles with chitosan are shown in Figure: 9. respectively. The average size of iron oxide nanoparticles is found to be 104 nm and that of chitosan modified iron oxide nanoparticles is found to be 157 nm.

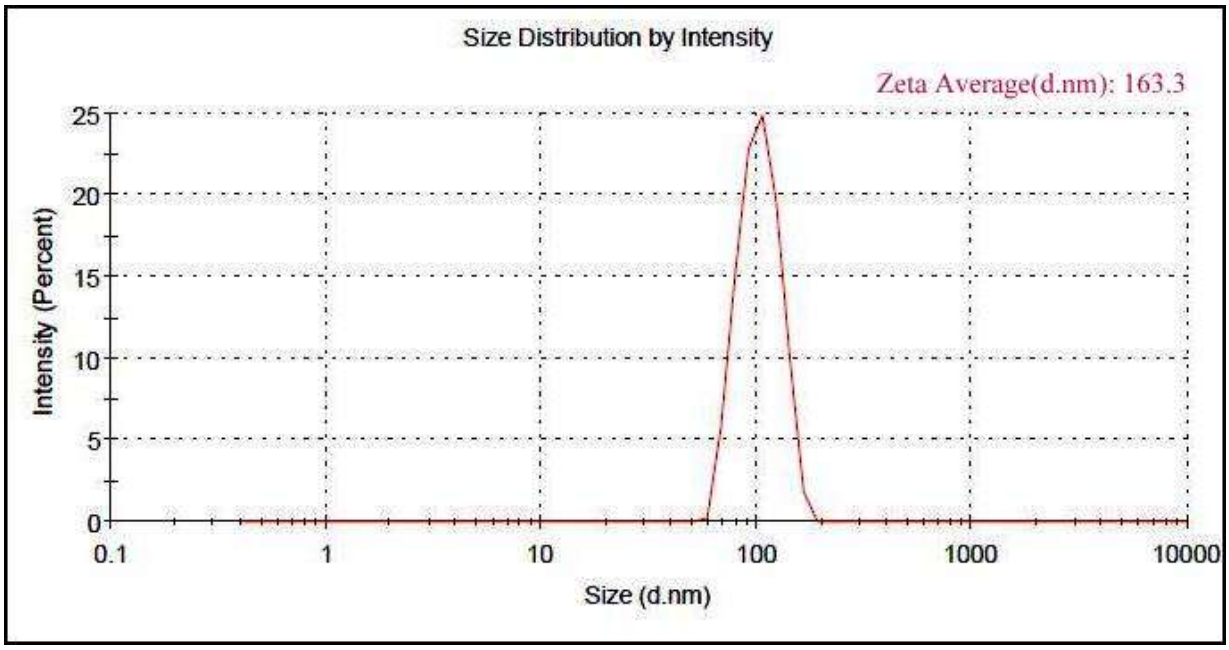

Figure: 8. Particle size distribution of Iron oxide nanoparticle

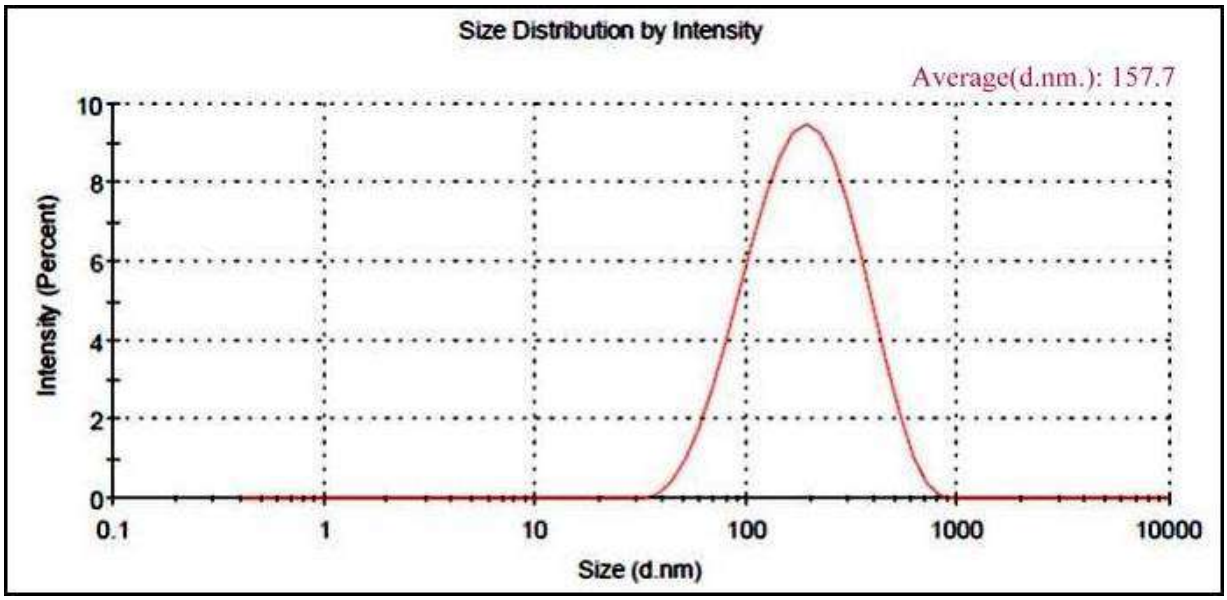

Figure 9: Particle size distribution of modified Iron oxide nanoparticle.

5.3.3. Zeta Potential Analysis:

Zeta potential is the potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed particle. Figure 10 and 11 show the zeta potential of

Iron oxide nanoparticle and chitosan modified Iron oxide nanoparticle. The zeta potential value of Iron oxide nanoparticle is -32.2 mV where as the zeta potential value of chitosan modified Iron oxide nanoparticle is 36 mV. The higher zeta potential value of chitosan modified iron oxide nanoparticles shows more stability than iron oxide nanoparticles. The analysis shows that the stability increases by the surface modification of the Iron oxide nanoparticle with chitosan.

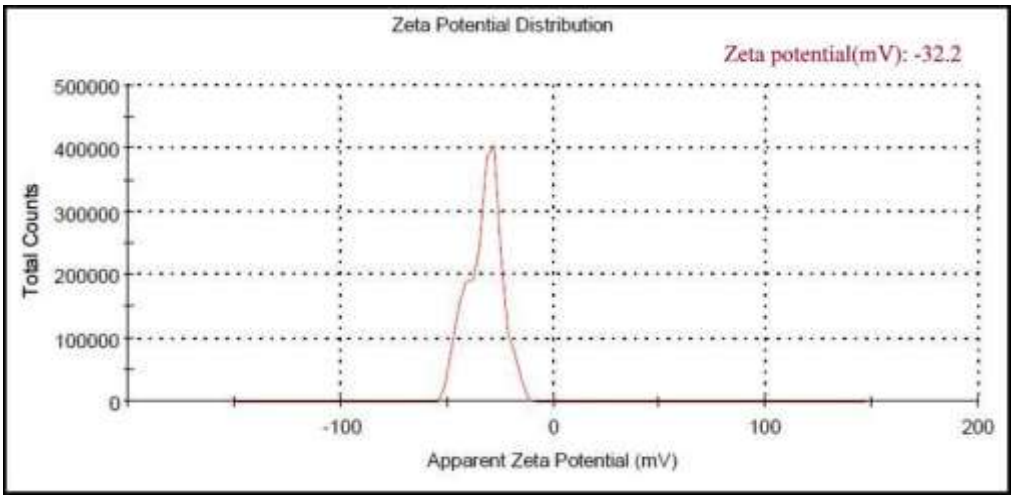

Figure 10: Zeta potential distribution of Iron oxide nanoparticle

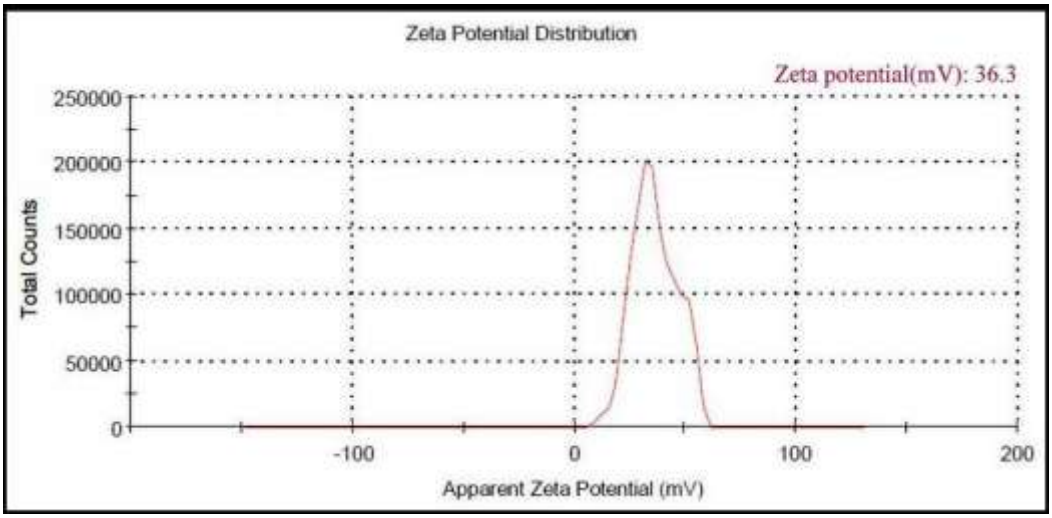

Figure 11: Zeta potential distribution of modified Iron oxide nanoparticle

5.3.4. XRD Analysis:

Figure 12 shows the XRD pattern of Iron oxide nanoparticle. The narrow peaks in the figure confirm the crystalline structure of iron oxide nanoparticles.

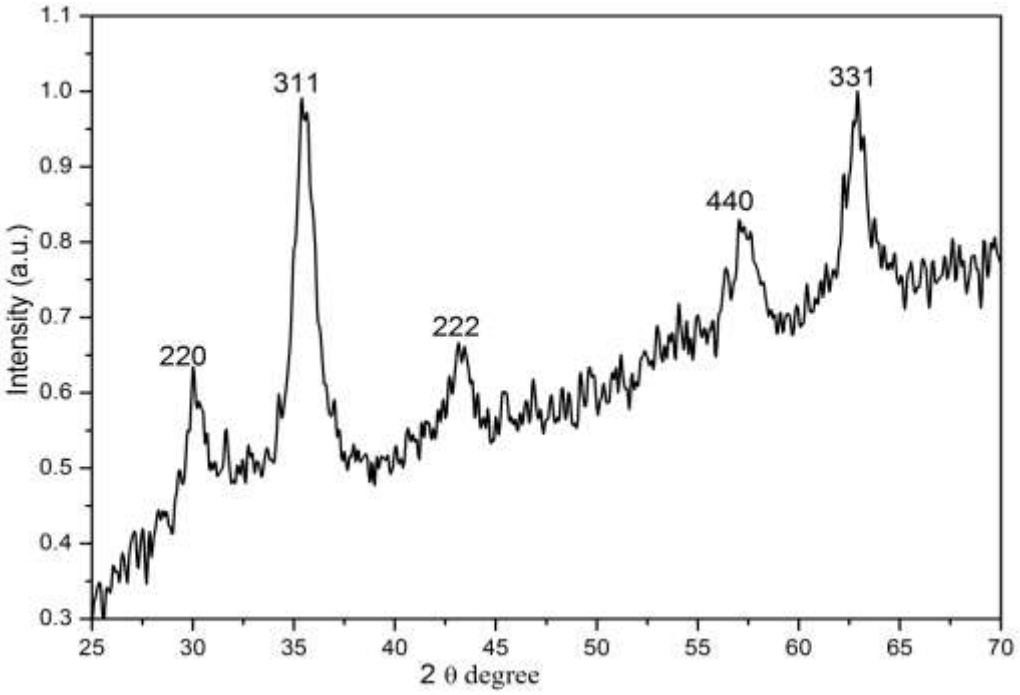

Figure 12: XRD pattern of Iron oxide nanoparticles

5.3.5. FTIR Result:

Figure 13 shows the FTIR spectra for Iron oxide and modified Iron oxide nanoparticles. The peaks at 519 cm^{-1} and 514 cm^{-1} confirm the presence of nanoparticles in the synthesized samples and surface modified samples. For chitosan, the amide-I vibration comes around 1676 cm^{-1} and for modified iron oxide nanoparticles it is shifted to 1708 cm^{-1}, which confirms the hydrogen bonding between oxygen molecule present in Iron oxide nanoparticles and hydrogen molecule present in chitosan. Moreover, FTIR study confirms the coating of chitosan on the surface of iron oxide nanoparticles.

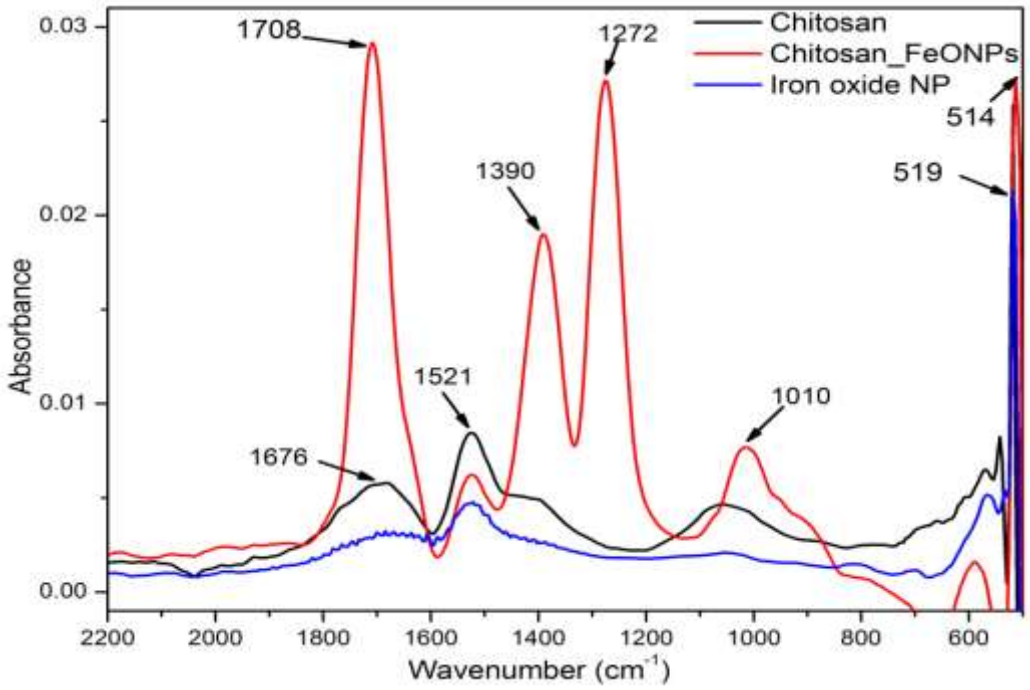

Figure 13: FTIR graph of iron oxide, chitosan and modified iron oxide

5.3.6. SEM Analysis:

Figure 14 shows the FE-SEM image of Iron oxide nanoparticle synthesized by chemical precipitation method, and figure 15 shows the FE-SEM image of modified Iron oxide nanoparticle. Size of the particles in iron oxide nanoparticles ranges from 8-20 nm and for surface modified iron oxide nanoparticles it ranges from 15-25 nm. The increase in the size of nanoparticles confirms the coating of chitosan to iron oxide nanoparticles.

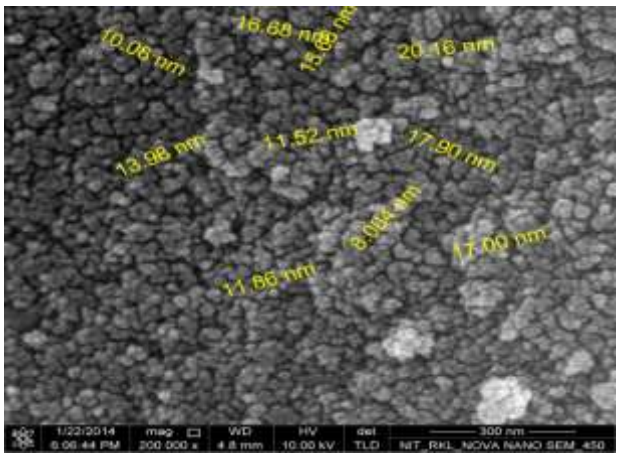

Figure 14: SEM image of Iron oxide nanoparticle

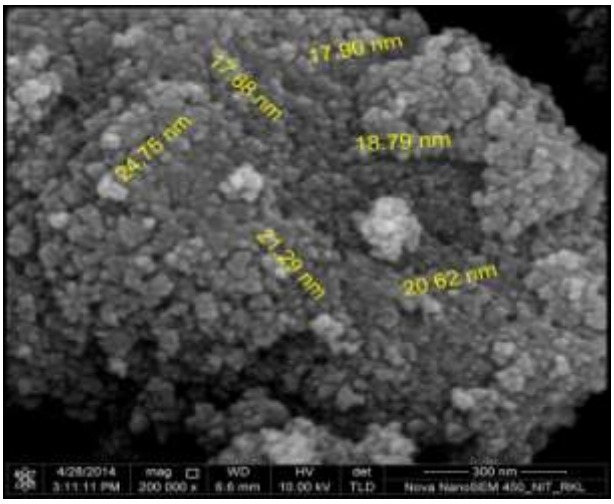

Figure 15: SEM image of modified Iron oxide nanoparticle

5.4. Growth kinetic study by iron oxide and modified iron oxide nanoparticles:

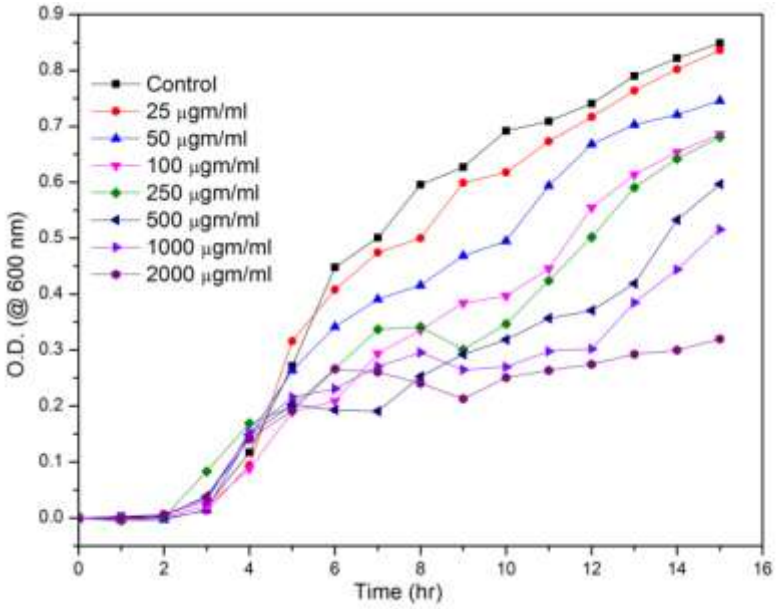

Figure 16: Growth kinetics of Bacillus subtilis in the presence of iron oxide nanoparticles

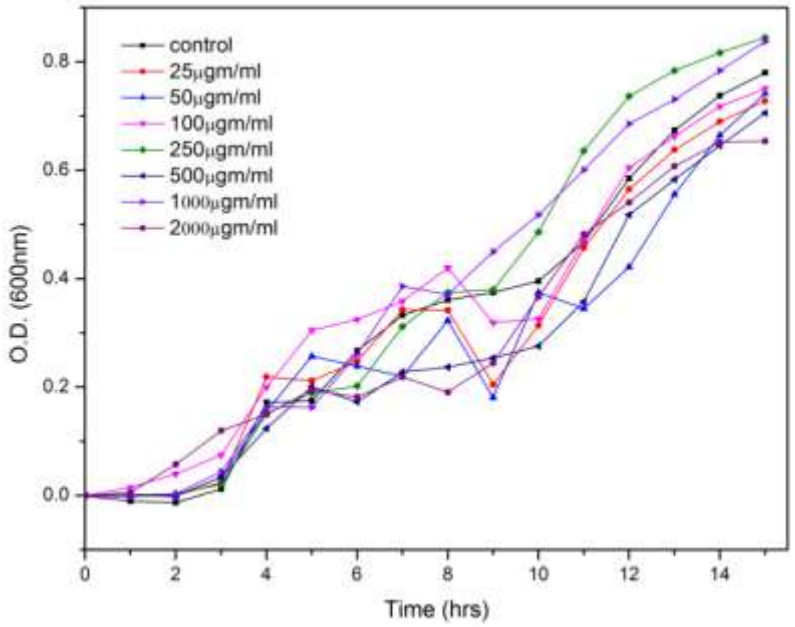

Figure 17: Growth kinetics of Bacillus subtilis in the presence of Chitosan modified iron oxide nanoparticle

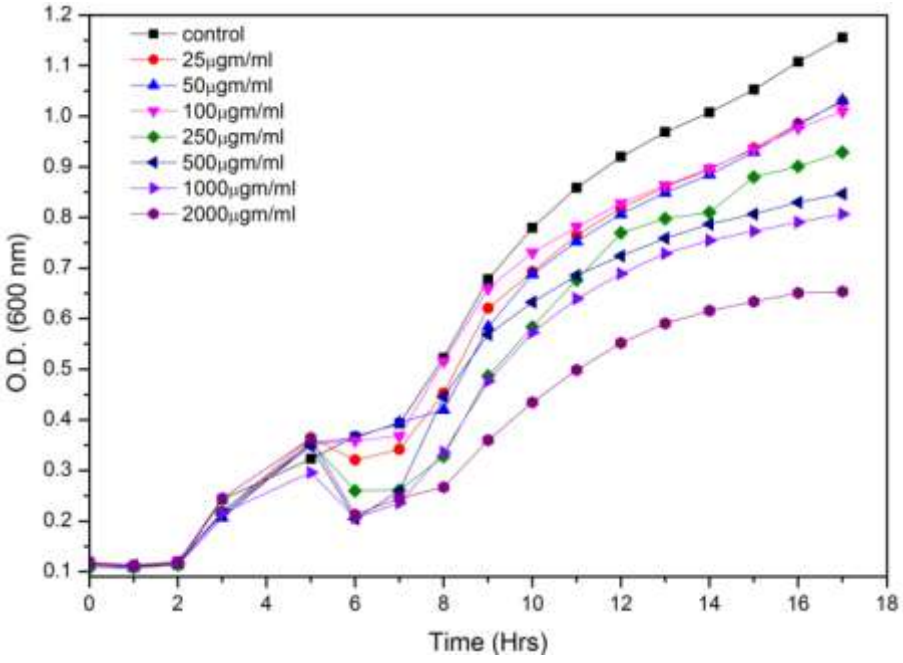

Figure 18: Growth kinetics of E. coli in the presence of iron oxide nanoparticle

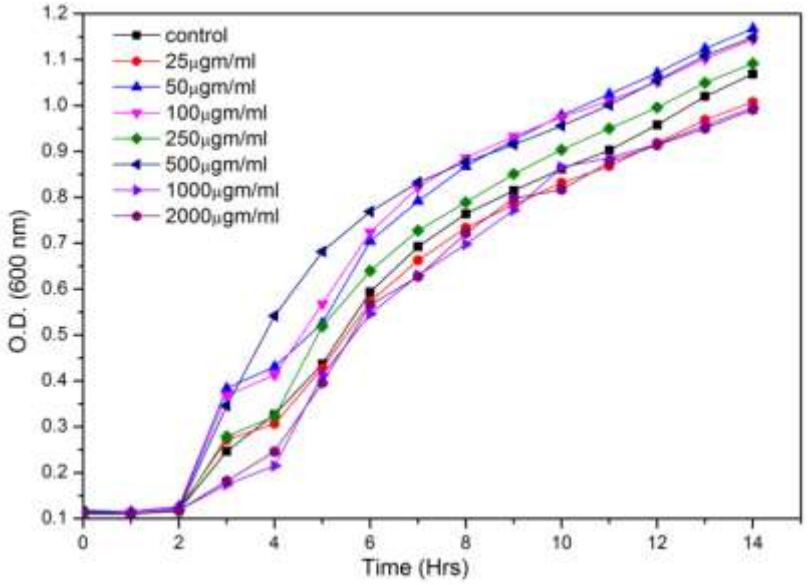

Figure 19: Growth kinetics of E. coli in the presence of Chitosan modified iron oxide nanoparticle

From the above graphs it is observed that in the presence of iron oxide nanoparticles growth of both *Bacillus subtilis* and *E. coli* strain is inhibited. But in the presence of chitosan modified iron oxide nanoparticle there is no effect on both the strains. Iron oxide nanoparticles show more antimicrobial activity to *Bacillus subtilis* than *E. coli*.

5.5 Phase contrast Microscopy:

The bacterial cells clump together when it comes in contact with the iron oxide nanoparticle where as there is no significant change in the presence of modified iron oxide nanoparticle.

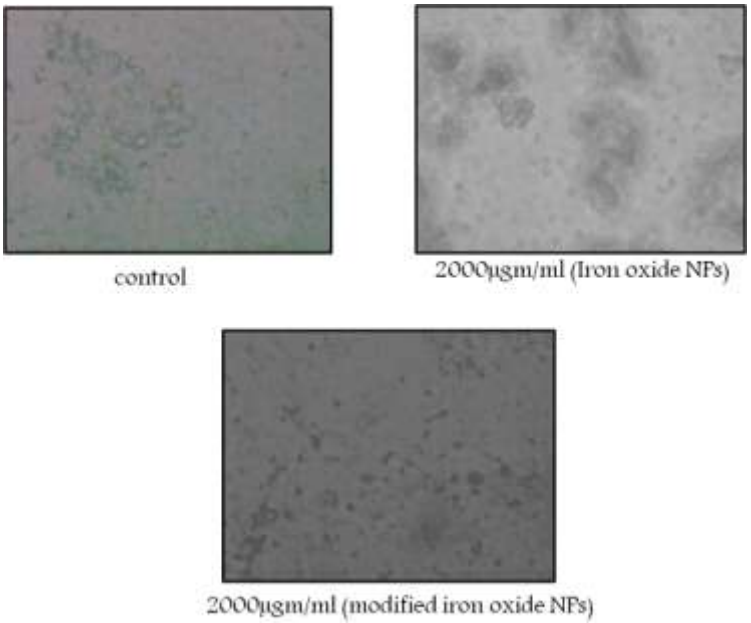

Figure 20: Phase contrast microscopy of E. Coli

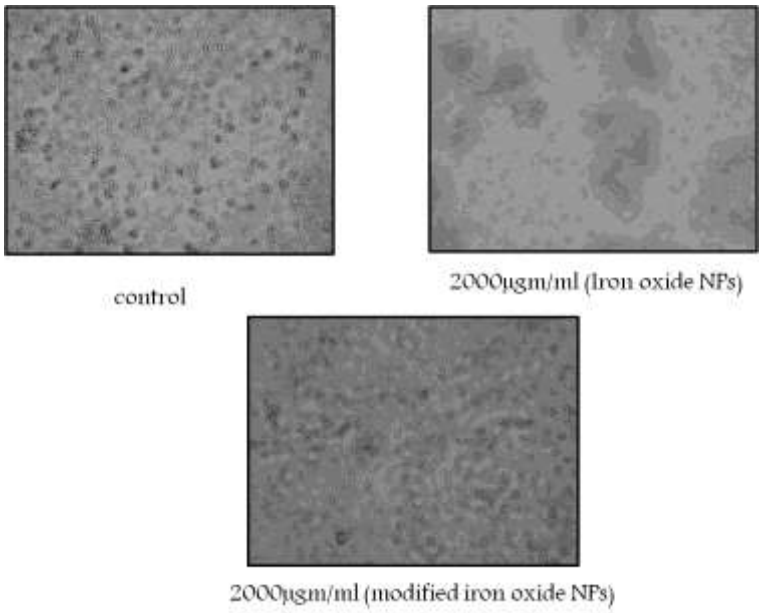

Figure 21: Phase contrast microscopy of Bacillus subtilis

Conclusion

The iron oxide nanoparticles were prepared by using co-precipitation method. Iron oxide nanoparticle showed the peak at 222 nm, whereas chitosan modified iron oxide nanoparticle showed peak at 268 nm. The shifting of peaks confirmed the coating of chitosan on the surface of iron oxide nanoparticles. The morphology of particles formed was studied by the FE-SEM analysis. Zeta potential analysis confirmed the increase in stability by surface modification whereas XRD analysis showed the crystalline nature of iron oxide nanoparticles. The iron oxide nanoparticles have the potential to inhibit the growth of both *Bacillus subtilis* and *E. coli*, whereas modified iron oxide nanoparticles doesn't possess this property.

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